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ORIGINAL PAPER

Drug resistance marker-aided genome shuffling to improve acetic acid tolerance in *Saccharomyces cerevisiae*

Dao-Qiong Zheng · Xue-Chang Wu · Pin-Mei Wang · Xiao-Qin Chi · Xiang-Lin Tao · Ping Li · Xin-Hang Jiang · Yu-Hua Zhao

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Abstract Acetic acid existing in a culture medium is one of the most limiting constraints in yeast growth and viability during ethanol fermentation. To improve acetic acid tolerance in *Saccharomyces cerevisiae* strains, a drug resistance marker-aided genome shuffling approach with higher screen efficiency of shuffled mutants was developed in this work. Through two rounds of genome shuffling of ultraviolet mutants derived from the original strain 308, we obtained a shuffled strain YZ2, which shows significantly faster growth and higher cell viability under acetic acid stress. Ethanol production of YZ2 (within 60 h) was 21.6% higher than that of 308 when 0.5% (v/v) acetic acid was added to fermentation medium. Membrane integrity, higher in vivo activity of the H^+-ATPase, and lower oxidative damage after acetic acid treatment are the possible reasons for the acetic acid-tolerance phenotype of YZ2. These results indicated that this novel genome shuffling approach is powerful to rapidly improve the complex traits of industrial yeast strains.

Keywords Ethanol fermentation · Genome shuffling · Acetic acid · Tolerance · *Saccharomyces cerevisiae*

D.-Q. Zheng and X.-C. Wu contributed equally to this work.

D.-Q. Zheng · X.-C. Wu (✉) · P.-M. Wang · X.-Q. Chi · X.-L. Tao · P. Li · X.-H. Jiang · Y.-H. Zhao
Institute of Microbiology, College of Life Sciences, Zhejiang University, Hangzhou 310058, Zhejiang, People's Republic of China
e-mail: mblab@163.com

Introduction

Yeasts are widely employed in the production of commercial compounds such as biofuels, yeast biomass, organic acids, amino acids, vitamins, and proteins. During the industrial process (i.e., ethanol fermentation) yeasts are continuously exposed to a myriad of environmental stress that can be bottlenecks for high fermentation efficiency [2]. Acetic acid existing in the culture medium, which chiefly results from bacterial contaminant or process reasons, is one of the most limiting constraints in yeast fermentation [10, 16]. The direct consequences of acetic acid stress include a decrease in intracellular pH, growth inhibition, and apoptotic cell death [17, 22, 29]. The genome-wide transcriptional analysis of *Saccharomyces cerevisiae* under acetic acid stress partly reveals how yeast cells adapt to and survive acetic acid stress [1, 4, 5, 15]. Membrane proteins such as Pma1p (H^+-ATPase) and Fps1p were noted to be particularly important for yeast to defend against this organic acid [19, 25]. However, there is no study reporting on the improvement of inherent tolerance against acetic acid stress in industrial *S. cerevisiae* strains.

Recently, an efficient technology named genome shuffling has been successfully used to improve stress tolerance, substrate uptake ability, and product yield in some microorganisms [12, 23, 28, 32]. This approach has the advantage of simultaneous genetic changes at different positions throughout the entire genome by recursive protoplast fusion or crossing. However, a crucial step, of how to select recombinants with a desired phenotype efficiently, remains a problem, and prohibits the widespread use of this technology.

In this study, drug resistance marker-aided genome shuffling was developed to improve the acetic acid tolerance and ethanol productivity of *S. cerevisiae* 308 rapidly.

Compared to the manipulation of genome shuffling described in previous reports [13, 28], this novel genome shuffling approach has the advantage of highly efficient selection of shuffled strains with the aid of G418- and Zeocin-resistance markers. By this approach, a mutant strain YZ2 with better growth and fermentation performance under the acetic acid condition was isolated after ultraviolet (UV) mutation and two rounds of genome shuffling. Finally, the mechanisms of tolerance improvement were investigated.

Materials and methods

Strain, medium, and plasmids

Diploid *Saccharomyces cerevisiae* strain 308 (received from Henan Tianguan Fuel Ethanol Co. Ltd.) is largely applied in production of fuel ethanol. Growth medium (YPD) contained (w/v): 1% yeast extract, 2% peptone, 2% glucose (pH 5.5). Corn mash containing 200 g glucose/l was used for fermentation medium (FM), pH 4.5. Any medium with acetic acid used in this study was titrated to pH 4.5 with either HCl or NaOH before autoclaving, except special requirement.

Plasmid pSH65, conferring Zeocin resistance, was received form Dr. Johannes H. Hegemann [11]. Plasmid pYK was constructed by insertion a 1.5-kbp PCR fragment (using the primers KanS: 5′-GTACTAGTCTGCAGGTC GACGGATC-3′ and KanA: 5′-GATGAATTCGAGCTC GTTTTCG-3′), containing G418-resistance gene from pFA6a-KanMX4 [11], into *Spe*I and *Sac*I sites of plasmid pYES2 (Invitrogen).

UV treatment

After cultivation in YPD at 30°C for 10 h, about 5 ml of the yeast (10^8 cells/ml) was irradiated with a Phillips TUV-30-W-254 nm Lamp for 35 s at a distance of 20 cm. The treated cells were kept in the dark for 2.5 h, spread on YPD agar plates and incubated at 30°C for 3 days. Colonies were picked, diluted and then spread on YNB plates (pH 4.5) with 0.3% (v/v) acetic acid for 36 h. The fast growing colonies were selected for genome shuffling.

Yeast transformation and plasmid curing

S. cerevisiae were transformed by LiAc/SS carrier DNA/PEG method [8]. To cure the plasmid pSH65 or pYK, yeast cells were cultivated in liquid YPAD (1% yeast extract, 2% peptone, 2% glucose and 0.04% adenine sulfate) medium at 30°C for 2 days, plated onto YPD plates containing 200 μg G418/ml or 50 μg Zeocin/ml and incubated at 30°C for 2–3 days. Colonies were screened for those that did not grow on YPD medium containing both 200 μg G418/ml and 50 μg Zeocin/ml by replica plating method.

Sporulation and crossing

The diploid cells of the initial population on each round of shuffling were grown to OD600 of 1 in liquid YPD medium containing 200 μg G418/ml or 50 μg Zeocin/ml and harvested by centrifugation. The resulting cells were washed twice with sterilized water and sporulated in the 200-ml flasks containing 50 ml of sporulation medium (1% NaAc, 0.2% yeast extract, 0.1% glucose, 0.2% KCl, pH 6.0) at 200 rpm and 28°C. The spores were purified and crossed by the method of Hou [13].

Deletion of *FPS1*

The *FPS1*-deletion cassette was made by PCR amplification of the loxP-KanMX4-loxP module from pUG6 with the primers FPS1S: 5′-TCGGTTGTTCTTCTTTATTA TTTACCAAGTACGCTCGAGGGGGGCTGGCTTAACT ATGC-3′ and FPS1A: 5′-CGAATCTTCTGATGA TGTGTCGGATAACCCAATAGAATCAATAGGGAGA CCGGCAGAT-3′. Correct integration was determined by PCR and sequences analysis using primers VIS: 5′-AGA GTTACGGCATAAGAAGT-3′ and VISA: 5′-AACCT CAGTGGCAAATCC-3′. Primers VFPS1S: 5′-TCATCCG ACGAAGGACGCTC-3′ and VFPS1A: 5′-TGTAGGT GTTTGAGGGTTCTGG-3′ were used to confirm the loss of *FPS1*.

Measurement of intracellular pH and analysis of proton efflux from cells

Strain 308 and YZ2 were grown to mid-log phase, harvested, and resuspended by YNB medium (pH 4) with 0 and 1% acetic acid at 30°C for 2 h before the test of intracellular pH and proton transport. The intracellular pH was measured with a spectrophotometer by the method of Bracey et al. [3]. To measure the proton efflux from yeast cell, fresh cells (300 mg) were washed three times with sterile water (4°C), suspended in 20 ml of distilled water (pH 6) at 25°C, and pH changes were recorded after the addition of cells.

Cell viability, membrane integrity, and intracellular reactive oxygen species accumulation

Stationary-phase cells of 308 and YZ2 were collected by centrifugation, washed three times with YNB, and then exposed to YNB medium with 0.5–2% (v/v) acetic acid at 30°C for 1–2 h. Cells were collected by centrifugation and

washed twice with 0.1 M PBS (pH 7.4) to remove residual acetic acid, before being stained with both 20 μg propidium iodide (PI)/ml and 10 μg fluorogenic dyes fluorescein diacetate (FDA)/ml or 10 μg dichlorodihydrofluorescein diacetate (DCFH-DA)/ml at 30°C for 1.5 h. The cell viability and reactive oxygen species (ROS) accumulation labeled by DCFH-DA were observed and imaged by laser scanning confocal microscope. Three fields of view from each coverslip were randomly chosen and the relative membrane integrity was calculated by the method of Wei [30].

Fermentation and metabolites

Anaerobic fermentations were carried out in 250-ml conical flasks covered with fermentation bungs at 33°C. The working volume was 100 ml and the shaker speed was 150 rpm. Yeast cells were pre-cultured aerobically in YPD for 15 h at 30°C and 0.025 g cells (dry weight) were transformed to fermentation medium. Glucose and ethanol were determined by an Aminex HPX-87H column (Bio-Rad, Richmond, CA) at 65°C with 0.6 ml/min eluent of 5 mM H_2SO_4. Detection was performed via differential refractive index detector.

Results and discussion

Improvement of acetic acid tolerance by drug resistance markers-aided genome shuffling

Genome shuffling has been presented as a novel and efficient technology to improve the complex phenotypes of microorganisms. During its operation, recursive protoplast fusion or crossing with multi-parental strains was used to implement recombination throughout the entire genome. The successes of this approach depend largely on the selection efficiency of recombinants with desired phenotypes. In the present study, a method (Fig. 1) was devised for the efficient selection of the hybrids from diverse population. The Vectors pSH65 (yeast centromere plasmid) and pYK (2 μ-based plasmid) used in this study contain different types of autonomously replicating sequences and can coexist in yeast cells. To estimate the holding stability of these plasmids during meiosis and sporulation, 30 transformants with pSH65 or pYK were subjected to tetrad analysis, which revealed that the recovery of pSH65 and pYK was 93 and 87.5%, respectively, even after meiosis, sporulation and subsequent germination. The G418- and Zeocin-resistance selective markers, powerful tools in genetic manipulation and breeding of *S. cerevisiae* [11, 21], facilitated the identification of the recombinant immensely because only the hybrids carrying both plasmids can appear on the drugs

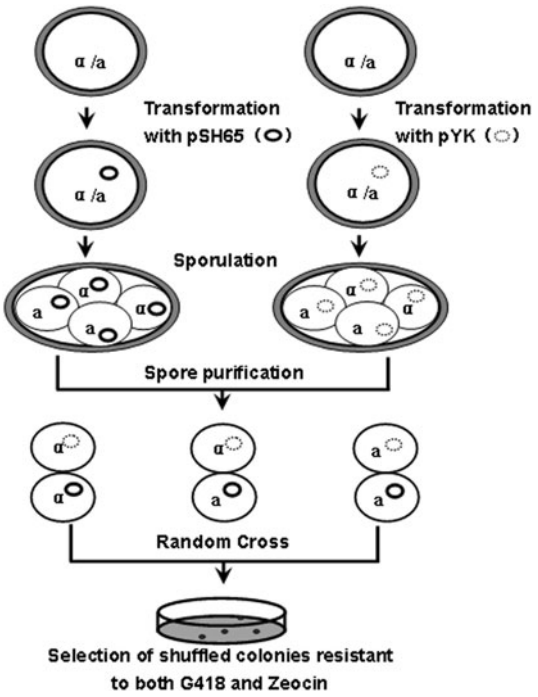

Fig. 1 Procedure of drug resistance marker-aided genome shuffling. The mixed culture of the initial diploid cells on each round of shuffling was divided into two: one was transformed with pSH65 and the other was transformed with pYK. The transformants were grown to OD600 of 1 in liquid YPD medium containing 200 μg G418/ml or 50 μg Zeocin/ml. The resulting cells were sporulated and the spores were mixed together to undergo crossing after spores purification. Cell suspensions from the crossing medium were diluted and plated on the drug selection plates (containing 200 μg G418/ml and 50 μg Zeocin/ml, pH 7). Hybrids that were resistant to both of the drugs appeared after 2 days

selection plates. Compared to selective methods (i.e., auxotrophic markers, micromanipulator, and inactivated parental protoplasts fusion) described in previous studies [7, 13, 14], this approach without the use of micromanipulator, ploidy tests, and damage to parental strains in this study was more straightforward to implement, less harmful to cells, and better suited for industrial yeast strains lacking feasible selection markers. This approach can also be applied to other microorganisms if the right drug resistance markers are available.

About 200 ultraviolet (UV) mutants of strain 308 that appeared first on the YNB plates with 0.3% (v/v) acetic acid were used as the starting population of genome shuffling. These UV mutants were mixed and divided into two: one was transformed with pSH65 and the other was transformed with pYK. The transformants were then subjected to sporulate and cross (Fig. 1). About 300 hybrids from the drug selection plates were mixed, diluted and then spread on YNB plates with 0.35% (v/v) acetic acid for primary screening of tolerance mutants. Fifty fast-growing colonies on the acetic acid selection

plates were chosen for shake-flask fermentation analysis (in triplicate flasks) to evaluate their fermentation rate and ethanol yield both with and without acetic acid stress. Five shuffled strains with best fermentation characteristics (two conditions were satisfied: (1) higher ethanol yield than that of the control strain 308 within a certain period under acetic acid stress; (2) the fermentation rate, ethanol yield and glucose/ethanol conversion are not inferior to that of strain 308 without acetic acid stress) advanced to the second round of shuffling. To recycle the drug selection markers, one of the two types of plasmids was cured in each of the five shuffled strains, as mentioned in the Materials and methods section, before sporulation in the second round of genome shuffling. The operation of sporulation, crossing and selection of hybrids was similar to that of the first round of genome shuffling, but the concentration of acetic acid used for initial selection was adjusted to 0.4% (v/v). Among the 50 colonies that grow faster on YNB plates with 0.4% acetic acid, the mutant strain YZ2 showed the best performance in the fermentation tests and was considered for further studies.

Improved tolerance of the selected strain YZ2 to acetic acid

Serial dilution assay was carried out to examine the resistance of the selected strain YZ2 and 308 to acetic acid. Significant differences between YZ2 and 308 were observed when 0.3, 0.4, and 0.45% (v/v) acetic acid was added to the YNB medium (pH 4.5) (Fig. 2b–d, respectively). In comparison, the growth performances of these two strains were similar on the control YNB plates (pH 4.5) (Fig. 2a).

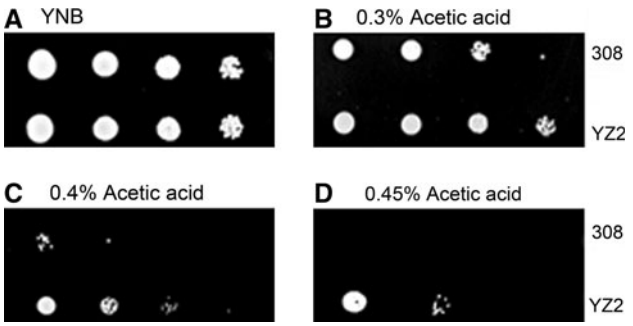

Fig. 2 Serial dilution assay was carried out to evaluate acetic acid sensitivity. Yeast cells were grown to the mid-exponential phase and collected by centrifugation. The cells (3×10^8 cells/ml) were 10-fold serially diluted and 3 µl of the indicated dilutions were then spotted onto YNB plates with **a** 0, **b** 0.3, **c** 0.4, and **d** 0.45% (v/v) acetic acid. Growth was monitored over 3–4 days at 30°C. Experiments were carried out in triplicate. One representative experiment is shown

Growth in liquid medium and ethanol fermentation

Under the anaerobic condition, the growth of YZ2 and 308 was similar in the YNB liquid medium (pH 4.5), but the lag phase of YZ2 was 20 h shorter than that of 308 ($p < 0.01$) in the YNB medium with 0.4% (v/v) acetic acid ($p < 0.01$) (Fig. 3). The final biomass of strain 308 and YZ2 under 0.4% acetic acid stress only reached 53% of that in the unstressed condition ($p < 0.01$) (Fig. 3). The dissociation of acetic acid in the cytoplasm was revealed to lead to rapid acidification of the yeast cell interior and long period of stasis [24]. To counteract this stress, yeast cells must expel proton via enhanced membrane H^+-ATPase activity [1, 15]. However, increased H^+-ATPase activity will result in significant loss of available energy for growth and other essential metabolic functions, which can explain the lesser biomass formation of YZ2 and 308 cultured in the YNB medium with 0.4% acetic acid compared with that in the unstressed condition.

The selected strain YZ2 and the control strain 308 were evaluated in a fermentation medium with 0 and 0.5% (v/v) acetic acid. The fermentation characteristics of YZ2 and 308 were similar under the unstressed condition and the glucose was almost exhausted within 45 h (Fig. 4a). Decreases in fermentation rate and ethanol yield were observed when 0.5% acetic acid was added to corn mash (Fig. 4b), but the shuffled strain YZ2 was noticeably more resistant to acetic acid. Compared to 308, YZ2 had a shorter lag phase and improved the ethanol production by 21.6% ($p < 0.01$) within 60 h (Fig. 4b). In the fuel ethanol industry, minor quantities of acetic acid are produced by *S. cerevisiae* during fermentation, but toxic concentrations may be produced by contaminated bacteria because pure

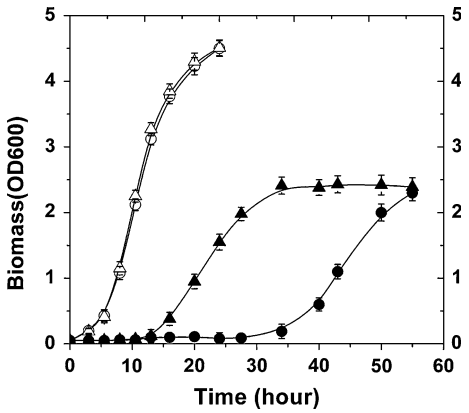

Fig. 3 Growth curve of strain 308 (*open circle* and *filled circle*) and YZ2 (*open triangle* and *filled triangle*) in YNB medium with 0 (*open symbols*) and 0.4% (*solid symbols*) acetic acid. Experiments were carried out in 150 ml conical flasks with 25 ml medium at 30°C. The initial OD600 was 0.05 and flasks were covered with fermentation bungs to keep anaerobic. The values represent the mean of three independent experiments and bars indicate SD

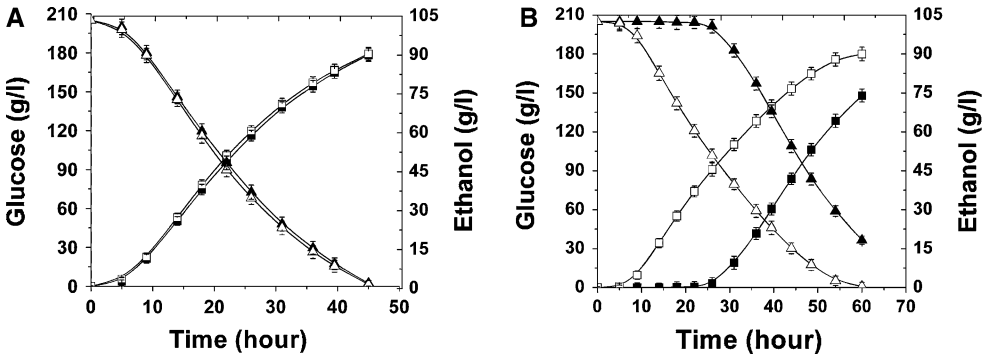

Fig. 4 Ethanol fermentation process of strain 308 (*solid symbols*) and YZ2 (*open symbols*) in (**a**) FM, pH 4.5 and (**b**) FM with 0.5% acetic acid, pH 4.5. The values represent the mean of three independent experiments and bars indicate SD

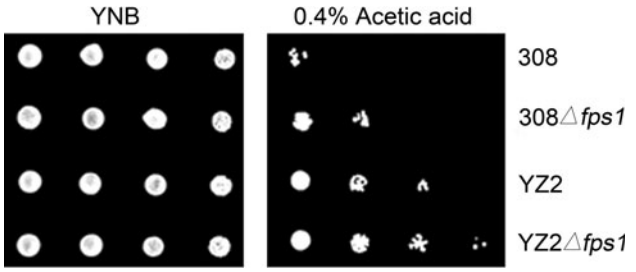

Fig. 5 Growth of 308, 308Δ*fps1*, YZ2 and YZ2Δ*fps1* on YNB plate (pH 4.5) with 0 and 0.4% (v/v) acetic acid is shown. Experiments were carried out in triplicate

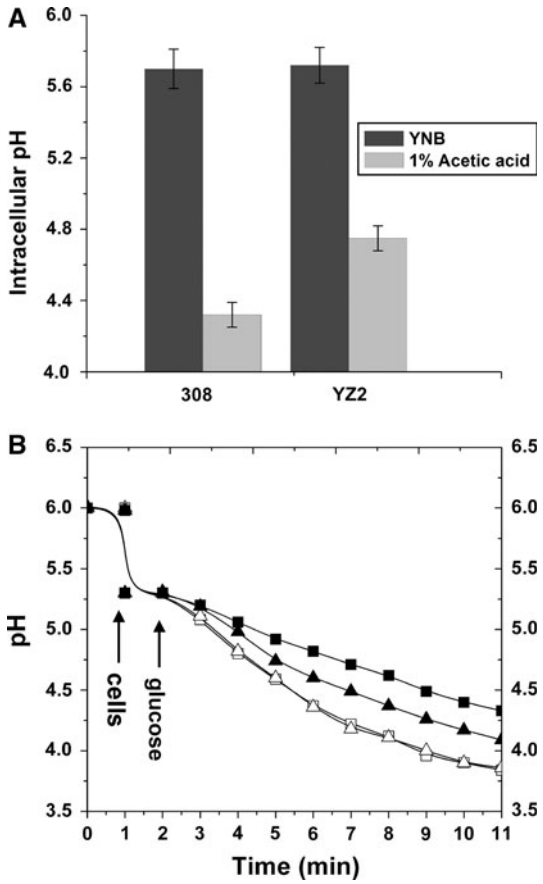

Fig. 6 **a** Intracellular pH of strain 308 and YZ2 after the treatment of YNB (pH 4) with 0 and 1% acetic acid stress is shown. **b** Analysis of proton efflux from cells to evaluate the in vivo Pma1p activity of 308 and YZ2. Cells and glucose (4 mmol) were added where indicated. Each data represents the average of two independent cultures each assayed in duplicate. Standard deviations were <5%

culture conditions are generally not practiced [10]. In addition, the lignocellulosic hydrolysate, considered to be a potential feedstock for ethanol production, contains high concentration of acetic acid due to pretreatment process [16]. Further studies are being taken to evaluate the lignocellulosic hydrolysate tolerance and fermentation performances at an industrial scale (in Henan Tianguan Fuel Ethanol Co. Ltd., China.) of strain YZ2.

Mechanisms of acetic acid tolerance improvement in the mutant strain YZ2

According to previous studies, the antimicrobial effects of weak organic acids depend largely on the form of its existence in the medium [22, 24]. At low pH, acetic acid (pK_a 4.75) exists substantially in the undissociated state (CH_3COOH), a form that readily diffuses across the cell membrane (mainly by passive diffusion) only to dissociate in the higher pH environment of the cytosol. Proton release has the potential to acidify the cytosol and inhibit many metabolic functions [22]. On the other hand, the acid anion tends to accumulate and cannot very readily diffuse from the cell. This high anion accumulation may generate abnormally high turgor pressure and influence free radical production, leading to severe oxidative stress in *S. cerevisiae* [25]. Moreover, the inhibition of growth by weak acids was proposed to be partly caused by their membrane disruption effects [15, 20]. In this study, comparative studies between 308 and YZ2 were performed in terms of the gene knockout of acetic acid channel, activity of H^+-ATPase, membrane integrity, and acetic acid caused oxidative damage to reveal the mechanisms of more acetic acid tolerance in YZ2.

The reports of Mollapour et al. [18, 19] have suggested that acetic acid stress could generate MAPK pathway

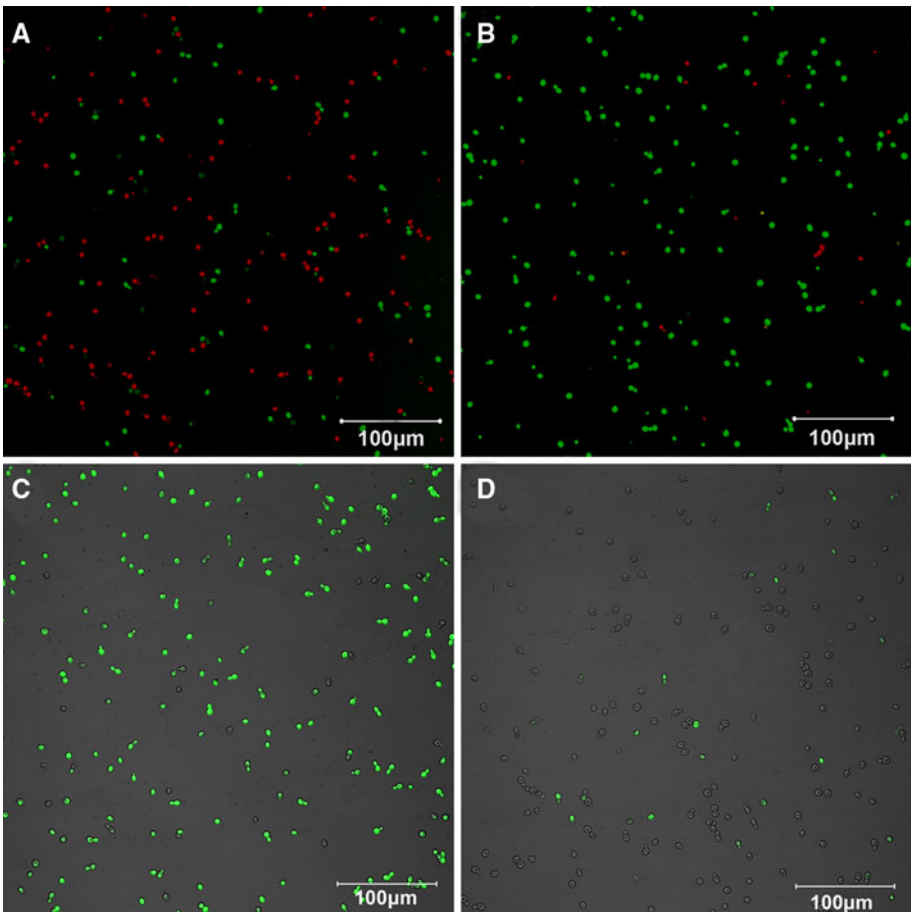

Fig. 7 **a** 308 and **b** YZ2 were stained with FDA/PI after the treatment of YNB (pH 3) with 2% (v/v) acetic acid for 2 h. Viable cells were stained *green* (*brilliant white* in the print version) with FDA and the cells that had lost membrane integrity were stained *red* (*greyish white* in the print version) with PI. The ROS accumulation in **c** 308 and **d** YZ2 was observed by staining with DCFH-DA after the treatment of YNB (pH 4) with 1% (v/v) acetic acid for 1 h

activated endocytosis of a plasma membrane aquaglyceroporin Fps1p, which enhances acetic acid tolerance by preventing passive diffusional flux of undissociated acetic acid into the cell. There is no doubt that the acetic acid tolerance of the parental strain would be enhanced if function inactivation would occur in Fps1p during the process of genome shuffling. Nevertheless, there was no sequence mutation in *FPS1* of YZ2 and no differences in the content of Fps1p (by immunoblot assay) were observed between strain 308 and YZ2 with and without acetic acid stress (data not shown). Furthermore, the gene knockout of *FPS1* improved the acetic acid tolerance not only in 308 but also in YZ2, which had not diminished the tolerance difference between YZ2 and 308 (Fig. 5). These results reveal that the difference of acetic acid tolerance between YZ2 and 308 was caused by other mechanisms, rather than Fps1p modification.

The plasma membrane H^+-ATPase (Pma1p), an ATP-driven proton efflux pump, is yet another important activity counteracting weak acid stress. Any acidification of the cytosol caused by the dissociation of weak organic acid could, in principle, be counteracted by increasing Pma1p-catalysed proton extrusion from the cell [9, 24]. Besides, multiple physiological processes of yeast, including nutrient transportation, were found to be closely related to intracellular pH, which is mainly regulated by Pma1p [24, 27]. In this work, YZ2 (pH 4.75) kept a higher intracellular pH than that of 308 (pH 4.31) under acetic acid stress (Fig. 6a) ($p < 0.05$), which suggested that YZ2 has a higher ability to relieve the acidification of cytosol and maintains a steadier physiological state under acetic acid stress. However, the Pma1p sequence of strain 308 and YZ2 was similar and no significant differences in the in vitro activity of Pma1p (in purified plasma membrane) were observed between strain 308 and YZ2 with and without acetic acid stress (data no shown). To reflect the more actual function of this proton pump, the in vivo activity of Pma1p between 308 and YZ2 was also compared by measuring the amount of proton efflux from the cells/min/mg fresh cells [26, 31]. Consistent with their intracellular pH, YZ2 and control strain 308 showed similar acidification power and lowered the pH value by 2.25 (0.15 nmol H^+/min/mg cells) without acid stress within 10 min after the cells was added, whereas YZ2 exhibited higher acidification power (0.058 nmol H^+/min/mg cells) compared to 308 (0.029 nmol H^+/min/mg cells) after treatment of 1% (v/v) acetic acid for 2 h ($p < 0.01$) (Fig. 6b). The higher in vivo activity of Pma1p may be related to the higher membrane integrity and cell viability of

YZ2 under acetic acid stress, as revealed by the examination of cells stained with FDA-PI (Fig. 7a, b). Specifically, the relative membrane integrities of YZ2 and 308 were 94 and 41%, respectively, after the treatment of 2% acetic acid for 2 h. It appears that the higher ability of YZ2 to keep membrane integrity under high concentration acetic acid stress may directly contribute to the function of membrane proteins and maintaining homeostasis under acetic acid stress.

Some previous reports have suggested that the membrane disruption effect of acetic acid is caused by its dissolution in the cell membrane, thus disturbing its structure and causing certain permeabilization [15, 20]. Thus, the changes in the properties of membrane and cell wall would affect the acid tolerance of the yeast. Nevertheless, the membrane integrity may have also suffered from acetic acid-caused ROS accumulation, which can generate numerous toxic reactants that rigidify membranes by cross-linking, disrupting membrane structure, and damaging membrane proteins [6]. As shown in Fig. 7c, d, the cells stained bright green (brilliant white in the print version) by DCFH-DA of 308 (80%) were much higher than that of YZ2 (13%) after the treatment of 1% acetic acid ($p < 0.01$), consistent with the results of membrane integrity and cell viability examination. Although the molecular mechanism of ROS formation with different levels in YZ2 and 308 should be investigated with further studies, the higher content of ROS in strain 308 could impose much worse oxidative damage on cell viability-related macromolecules (lipid, protein and nucleic acid) and increase the risk of ROS-induced cell death.

In conclusion, the drug resistance marker-aided genome shuffling technique was explored to improve the acetic acid tolerance and ethanol productivity of *S. cerevisiae*. With this technique, a shuffled strain YZ2 was obtained which enhanced cell viability and ethanol fermentation performance significantly under acetic acid stress. Membrane integrity, higher in vivo activity of the H^+-ATPase, and lower oxidative damage after acetic acid treatment may have played a pivotal role for YZ2 in the acquisition of tolerance to acetic acid. These results demonstrate this novel genome shuffling approach can be an efficient tool to improve valuable commercial traits in industrial *S. cerevisiae* strain.

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